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13. ABSTRACT *(Maximum 200 words)*

Our research will lead to a better understanding of the developmental, physiological, and biochemical roles of endogenous and environmental estrogens in breast cancer causation, prognosis and treatment. **We have found in ER-α minus mice a uterine lactoferrin mRNA response to the catechol estrogen, 4-hydroxyestradiol, but not to estradiol. We hypothesize that the putative 4-hydroxyestradiol receptor will elicit additional responses distinct from estradiol and the classic ER-α and ER-β proteins.** Using this mouse model system we propose to characterize the 4-hydroxyestradiol response and the putative 4-hydroxyestradiol_receptor. Specifically, we propose to: Aim #1 Characterize lactoferrin mRNA responses to 4-hydroxyestradiol_in ER-minus mice; Aim #2 Characterize the putative 4-hydroxyestradiol receptor in uteri from ER-minus mice; Aim #3 Compare the specificity of the responses to 4-hydroxyestradiol with those of estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, and tamoxifen in ER-minus mice; and Aim #4 Clone the putative 4-hydroxyestradiol_receptor, which is different from both ER-α and ER-β. This new "estrogen" receptor is of major importance because of its potential involvement in novel pathways of estrogen responsiveness that may better explain estrogens' roles in breast tumor progression, prognosis, and therapy.

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Dennis B. Lubln 3/22/99

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Introduction:

Importance of Catechol Estrogen Responses in Human Breast Cancer

Catechol estrogens have been studied extensively since enzymes capable of synthesizing them *in vivo* were discovered in the late fifties and early sixties. Their chemistry, biology, and potential functions have been well reviewed over the years (3-7). We will briefly review the importance and significance of catechol estrogens here. Catechol estrogens' structure and metabolism are diagrammed in **Figure 1** below.

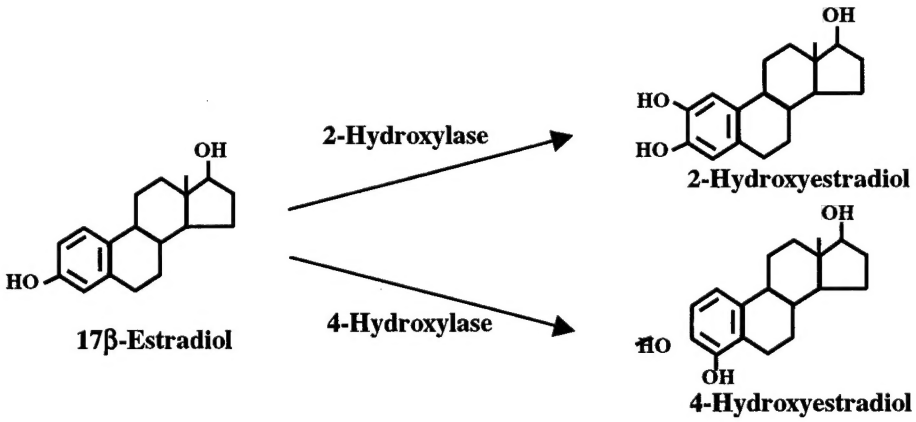


Figure 1. Metabolic conversion of 17β-estradiol to catechol estrogens: 2-hydroxyestradiol and 4-hydroxyestradiol.

Investigating the overall mechanism of estrogens' actions through all their receptors is crucial in understanding breast tumor progression, prognosis, and therapy. Catechol estrogens have been found to be tumor associated in many studies of human breast cancer (Refs. 74-101), human uterine cancer (8), and hamster kidney tumor formation (9,10). Their biological function is thought to be highly localized because of their rapid clearance from plasma (11). Ratios of 2- and 4-hydroxyestradiol concentrations vary between tissues because of differences in rates of synthesis and metabolism to their methoxy derivatives (6-8,12). The tissue specificity data and the data presented in Preliminary Studies (102) strongly support the hypothesis that catechol estrogens are more than simple catabolic products destined for excretion, but have potentially important roles in human physiology and disease as ligands for as yet uncharacterized receptors. We hypothesize that the existence of the putative 4-hydroxyestradiol receptor has been masked, mainly because catechol estrogens will also bind and activate the classic ER protein (13,14), which is present at relatively higher concentrations in humans.

Candidate Proteins Involved in Non-Classical Estrogen Responses

Candidate proteins fall into one, or possibly more, of the categories of estrogen response proteins listed in **Table 1** on the next page. Proteins #1 - #12 are listed below the Table as candidates for the putative 4-hydroxyestradiol receptor (4OHER), listed briefly for completeness, and/or listed for their potential as confounding factors in characterizing the putative 4-hydroxyestradiol receptor.

Table 1: Response Found with a Biologically Active "Estrogen" that is Mediated by a Particular Type of Potential Estrogen Response Protein

	Types of Estrogen Response Proteins				
"Estrogens"	A1(ER)	A2(ASER)	B1(NCER)	B2(4OHER)	B3(MXCR)
17β-Estradiol	Yes	?	?	No	No
4-Hydroxyestradiol	Yes	?	?	Yes	No?
Methoxychlor (metabolites?)	Yes	?	?	No?	Yes
[Other Natural Estrogens: Estrone, Estriol, Other Catechol estrogens, Flavones, Phytoestrogens. **Synthetic Estrogens:** Diethylstilbestrol, Tamoxifen; Kepone, Some Other Pesticides, Insecticides, Herbicides, etc.]	Yes	?	?	?	?

Five Types of Estrogen Response Proteins

A1. Classical Full Length Estrogen Receptor (ER) Protein

Examples; Nuclear receptor from ER gene, also called ER-alpha membrane (non-genomic) receptor?, Welshons' non-translocatable receptor?,

A2. Non-classical Alternately Spliced Estrogen Receptor (ASER) Proteins

Examples; Alternate splice form of A1, Shupnik's alternately spliced pituitary ER mRNA coding for a novel protein? and potential alternately spliced protein, artifact in ER-α minus mice?

B1. Non-classical Estrogen Response (NCER) Proteins

Examples; Gustafsson's ER-beta, Type II ER, tamoxifen receptor, other catechol estrogen receptors / orphan receptors (G-protein / 7 transmembrane or steroid receptor super families)?, Welshons' non-translocateable receptor?, c-erbB2, membrane (non-genomic) receptor?

B2. Putative 4-Hydroxyestradiol Receptor (4-OH ER) Protein

B3. Putative Methoxychlor Receptor (MXCR) Protein

Includes receptors for possible methoxychlor metabolites that may be mediating responses.

Table 1 Footnote:

Description of potential estrogen response protein candidates (#1-#12)

1) Classic, wild-type, full length ER (or Type A1) protein

In general, non-classical, genomic estrogen response pathways potentially involve at least one of the non-ER proteins (described below). Additional, non-classical, non-genomic estrogen response pathways (15-18) that may be mediated through the classic ER protein or other non-ER proteins are not part of this proposal.

2) Catechol estrogen "receptor(s)" - (Putative 4-hydroxyestradiol receptor?)

Catechol estrogens are produced by the hydroxylation of the 2 and 4 positions of estradiol (see Figure 1, page 20). While catechol estrogens bind to the classic ER with high affinity (14,15), 17b-estradiol does not bind to a partially purified membrane "catechol estrogen" receptor (19). Because of this lack of 17b-estradiol binding, the fascinating literature suggesting novel functions for catechol estrogens (reviewed earlier) and most importantly, the responses seen with 4-hydroxyestradiol in the ER-α minus mice (see Preliminary Studies section), we also hypothesize that a unique receptor for catechol estrogens may exist. Methoxychlor metabolites are known that contain the catechol structure of two adjacent hydroxyls on an aromatic ring (20). Because of the similarities in chemical structure, it is possible to speculate that a methoxychlor metabolite might also bind to a putative catechol estrogen receptor. Proposed competition studies would allow us to examine this question (see ***Specific Aim #2****).*

3) Jan-Ake Gustafsson's Estrogen receptor - beta

ER-beta, a novel member of the steroid receptor super family, has been cloned by Gustafsson et al. (personal communication, 21,22). Homology of ER-b to the classic estrogen receptor (now termed ER-alpha) is 17% in the N-

terminal region, 96% in the DNA-binding domain, 29% in the hinge region, and 55% in the estradiol-binding domain. ER-beta expressed in rabbit reticulocyte lysates binds to estradiol with an affinity of 0.6 nM. It is 485 amino acids long with a molecular weight of 54,000 daltons and can activate transcription through an estrogen response element. ER-b is found in rat prostate from which it was cloned, primarily in the epithelial cells (ER-a is primarily in prostate stromal cells), in the uterus, and in most other tissues but not in mammary gland or in the breast cancer cell lines, MCF-7, ZR75 or T47D. In uterus ER-beta seems to be present at 5-10% of the concentration of classic ER. We are now checking, as is likely, to see if this explains the residual estradiol-binding activity found in the ER-α minus mice. Unique binding characteristics to distinguish ER-alpha and -beta have not yet been released. The lack of inhibition by estradiol and ICI of the 4-hydroxyestradiol response excludes ER-beta as a candidate for the putative 4-hydroxyestradiol receptor (21,22, 102 – see Appendix).

4) Orphan receptors of the Steroid Receptor Superfamily, ERR1 and ERR2

The estrogen-related receptors 1 and 2 (ERR1 and ERR2) have limited homology to the ER gene and are not reported to bind estrogens (catechol estrogens, methoxychlor and kepone are not mentioned) (23). Their native ligands are unknown (hence the name orphan receptors) and, except for their homology to ER, they have no known functional connection to estradiol. Because a response has been observed to 4-hydroxyestradiol, methoxychlor and kepone in ER-α minus mice, we must seriously consider that the putative receptors to which they bind may be orphan members of the steroid receptor superfamily. Homology to a known steroid-binding gene family would allow the screening of cDNA libraries under low stringency conditions and would enable additional candidate genes for the putative methoxychlor receptor, like ERR1 and ERR2, to be cloned, expressed, and analyzed (see ***Specific Aim #4****).*

5) Membrane estrogen receptor or binding protein

Despite nearly 30 years of reports it is only recently that a "membrane estrogen receptor" has gained qualified acceptance (24). Evidence for membrane estrogen receptors come from reports based on biochemical isolation (25-27), immunocytochemistry (28), fluorescent labeled estradiol (29,30) and estrogen immobilization on an inert support (31). It is not clear that these all represent the same protein because of the widely divergent techniques used in the analyses.

6) Type II Estrogen Receptor

This low affinity estrogen binding protein with a binding affinity lower than the classic ER has been the subject of recent successful purification reports (32,33). The type II ER is 73 kd in size with a Kd for estradiol of 24 nM. The ER-α minus residual uterine estradiol-binding activity (see #10) appears not to be Type II ER because of its 0.2 nM K_d for estradiol (1,2).

7) Putative Tamoxifen Receptor

There exists a tamoxifen-binding protein that is distinguished from the classic ER on the basis of binding specificity studies (34-36). Estradiol has little or no affinity for this "tamoxifen" receptor (34). In preliminary experiments we have not found any biological responses to tamoxifen in the ER-α minus mice; unlike methoxychlor, kepone or 4-hydroxyestradiol, tamoxifen does not induce lactoferrin mRNA.

8) Welshons' non-translocatable cytoplast ER

Currently there is a form of estrogen receptor that is not found in the nucleus after estrogen stimulation in estrogen-sensitive breast cancer cells (37). Present evidence suggests a post-translationally modified ER, perhaps positioned to mediate non-classical, non-genomic effects.

9) c-erbB2, also called neu or HER2

There has been a surprising recent report, unconfirmed as far as I know, that the protooncogene, c-erbB2 binds estradiol with a 2.7 nM K_d (38). This protein is a 185 kd transmembrane glycoprotein similar to the EGF receptor. Estradiol is reported to activate the tyrosine kinase activity of c-erbB2 and down regulate this protein.

10) Residual Uterine Estradiol-Binding Activity Found in ER-α Minus Mice (Artifact?)

Recent work from our own lab has demonstrated that Gustafsson's ER-beta is the likely source of most of the 5-10% residual uterine estradiol-binding activity (1,2) and not ER-a, since that is the level that has been reported for ER-beta in the uterus (21,22). No classic estradiol responses potentially mediated through this residual activity have yet been found (1,24) although differential display suggests they exist (data not shown).

11) Shupnik's translation product (ASER) from alternately-spliced pituitary ER mRNA

This alternately spliced form of ER mRNA is present in high levels in rat pituitary (39,40). We have made the necessary oligonucleotides and will use them to look for an ASER mRNA in the ER-α minus uterus and pituitary. (No resources were requested for these studies in this proposal.)

12) A novel NCER protein

*A completely novel NCER protein might be detected in the homozygous ER-α minus mice. A lack of homology to other known receptors when attempting to clone a completely novel, putative 4-hydroxyestradiol receptor (**See Aim #4**) would require isolation and cloning techniques that utilized affinity chromatography or expression cloning.*

Body:

Results:

Cell-Type Specific Uterine Expression of Lactoferrin (LF) Gene in ER-α minus Mice Exposed to Estradiol-17β, Methoxychlor, Kepone, or 4-Hydroxyestradiol (4-OH-E_2)

Early experiments were performed to examine whether uteri of homozygous ER-α minus mice mutated for the ER gene respond to various estrogenic compounds (**102, see Appendix for a copy of paper**). Estrogenic compounds examined were estradiol-17β (primary estrogen), methoxychlor (41-43, 102), kepone (44), and 4-hydroxyestradiol a catechol estrogen. Additional experiments were carried out using tamoxifen and ICI-182,780 (antiestrogens), and ICI-182,780 in conjuction with estradiol-17β or 4-hydroxyestradiol.

Surprising and exciting results were observed. Consistent with previous findings (2), treatment with estradiol-17β failed to induce the LF gene in the uterus of ovariectomized ER-α minus mouse. However, to our surprise, treatments with 4-OH-estradiol-17β, methoxychlor (**103**), or kepone induced the expression of this gene in the uterine epithelium of ovariectomized ER-α minus mice; the expression was most prominent in the luminal epithelium. **These results have been verified via quantitative PCR (102,103)**. Treatment with the ER-specific antagonist, ICI-182,780 alone (45,46), did not influence this uterine gene. However, again to our surprise, ICI-182,780 did not interfere with the induction of the LF gene by 4-OH-estradiol. Because estradiol is non-responsive, it also was used in competition experiments with 4-OH-estradiol-17β, where it was also unable to interfere with LF induction.

Additional control experiments with the COMT (catechol O methyl transferase) inhibitor, U-0521, (47) have shown that it does not stimulate LF mRNA induction. This is important because catechol estrogens are capable of inhibiting COMT, which is necessary for the inactivation of dopamine, a catecholamine. Increased local dopamine levels might induce LF mRNA synthesis through the catecholamine receptors. The lack of effect by a COMT inhibitor indicates that 4-hydroxyestradiol is not acting indirectly to stimulate LF mRNA induction. In addition, since COMT alone does increase active dopamine levels, the likelihood is decreased that the lactoferrin response is mediated through a catecholamine receptor.

These results suggest the presence of a signaling pathway that is not mediated via the conventional ER, but rather by the putative 4-hydroxyestradiol receptor. (The competitive inhibition results of 4-hydroxyestradiol with estradiol or ICI-182,780 are very important because they tend to exclude any confounding problems with the ER-α minus residual uterine estradiol-binding activity / Gustafsson's estradiol-binding ER-beta (21,22).) These results suggest that an acute treatment with 4-hydroxyestradiol induces the LF gene in a cell-type specific manner in the mouse uterus. In recent results, we have confirmed the original results with lactoferrin mRNA and also observed that glucose 6 phosphate dehydrogenase is also induced in ERa-minus mice uterus by methoxychlor but not by estradiol (103).

Hypothesis/Purpose:

4-Hydroxyestradiol and other biologically important estrogens work, not only through the classic ER protein, but also through their own unique non-ER receptors. These receptors can be readily characterized in an ER-α minus mouse background. Specifically, estrogen responses in the uterus and mammary gland are not mediated exclusively by the classical ER protein but in addition by specific types of non-classical estrogen response (NCER) proteins. Characterization of estrogen responses and NCER proteins in ER-α minus mice will lead to a fuller understanding of the physiological and pathological roles of all estrogens in breast cancer.

Technical Objectives/Specific Aims:

The first estrogen responses found in ER-α minus mice are to 4-hydroxyestradiol (a catechol estrogen), methoxychlor, and kepone. We are proposing initially to characterize the 4-hydroxyestradiol response and the putative 4-hydroxyestradiol receptor, because of the importance of catechol estrogens in breast cancer.

Specifically, this grant proposes experiments to:

Specific Aim #1. Characterize lactoferrin mRNA responses to 4-hydroxyestradiol in ER-α minus mice.

Specific Aim #2. Characterize the putative 4-hydroxyestradiol receptor in ER-α minus mice.

Specific Aim #3. Compare the specificity of the responses to 4-hydroxyestradiol with those of estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, and tamoxifen in ER-α minus mice.

Specific Aim #4. Clone the putative 4-hydroxyestradiol receptor (4OHER).

Experimental Design / Methodology:

Hypothesis #1: Lactoferrin will respond to 4-hydroxyestradiol through a receptor-mediated pathway.

Specific Aim #1.

Characterize lactoferrin mRNA responses to 4-hydroxyestradiol in ovariectomized (ovex), ER-α minus mice.

1a. *In vivo* uterine characterization of lactoferrin induction via *in situ* hybridization analysis.

i. Dose response by within-run computer image analysis (Ambion)

ii. Specificity by comparison of 4-hydroxyestradiol responses with these 5 compounds: estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, and tamoxifen

1b. *In vivo* tissue specificity and quantitation of lactoferrin mRNA response to 4-hydroxyestradiol via *in situ* hybridization analysis and quantitative PCR. Examine uterus and mammary gland.

1. Rationale:

1a. Characterization of lactoferrin mRNA response to 4-hydroxyestradiol provides potential clues about purification and function of the putative receptor for 4-hydroxyestradiol. A dose response curve will be obtained *in vivo* then *in vitro* to get an estimate of the physiological/pharmacological concentration range of 4-hydroxyestradiol. This will give us a rough idea about the putative receptor binding constant. Two of the six compounds, 4-hydroxyestradiol and methoxychlor, were chosen because they elicit responses in ER-α minus mice. The catechol estrogen, 4-hydroxyestradiol, is made from estradiol by a hydroxylase and methoxychlor is known to be metabolized to a catechol (20). 2-Hydroxyestradiol is usually found in varying rations where 4-hydroxyestradiol is made (See **Figure 1**). 4-Methoxyestradiol is considered to be an inactivated form of 4-hydroxyestradiol. However, this 4-methoxy form is less active only with ER; it is not clear if it will also be inactive with 4OHER. 2-Hydroxyestradiol and 4-methoxyestradiol if unresponsive, as well as estradiol, tamoxifen, and ICI-182,780 will be used in competition studies to roughly determine the specificity of the receptor response.

1b. Determination of lactoferrin mRNA expression in various tissues after 4-hydroxyestradiol exposure will provide information about the receptor's tissue distribution. Tissues to be checked express lactoferrin (48-50). Quantitation will be by quantitative PCR. If possible, a response in a primary uterine culture would rule out an indirect endocrine action of 4-hydroxyestradiol.

1. Experimental Design / Methods / Expected Results / Potential problems:

1a. Steroids are from Steraloids Inc. (Wilton, NH); ultrapure methoxychlor is from Radian (Austin, TX). *In situ* hybridization will be performed to determine the dose response of methoxychlor induction of uterine lactoferrin mRNA. Initial doses will be one oral dose in oil followed by a 24-hour interval of 500 ng, 5 ug, 50 ug, 500 ug, and 5 mg per 30 gram mouse. If it does not appear saturable, we will try to go to 50 mg. A time course, from 2 hours after the last optimal dose from above, will be extended out until values return to background. This will determine the rate of the reversibility of the response. Estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, tamoxifen, and ICI-182,780 will be used individually (doses determined by dose response curve) and in combination (at 100 fold excess) with 4-hydroxyestradiol to determine if they will stimulate or inhibit the response and are working through the same receptor mechanism.

1b. The listed tissues express lactoferrin (48-50) and will be examined by *in situ* hybridization for responses at the optimal dose found in uterus. These optimally responding tissues will be examined for dose response to roughly compare their binding and response parameters with the uterine receptor.

Hypothesis #2: There is a unique receptor for 4-hydroxyestradiol distinct from ER-α and ER-β.

Specific Aim #2.

Characterize the putative 4-hydroxyestradiol receptor in uteri from ER-α minus mice.

2a. Localization by subcellular fractionation

2b. Analyze for saturable binding, binding affinity, and ligand specificity

2. Rationale / Experimental Design / Methods / Expected Results:

A receptor by definition must display low capacity, saturability, and specificity with a binding affinity consistent with its dose response curve. Thus, binding studies with [6,7-^{3}H]-4-hydroxyestradiol will be performed by whole cell uptake in primary uterine culture. Competition studies will be performed with estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, tamoxifen, and ICI 182,780 to demonstrate specificity. 2-Hydroxyestradiol and 4-methoxyestradiol will be used because they are estradiol metabolites and are found wherever 4-hydroxyestradiol is found. Similarly, methoxychlor is also known to have catechol and methoxy metabolites (20). It is possible that catechol estrogens and methoxychlor may share the same non-ER receptor as well as being capable of activating ER. Unique binding specificity will allow us to utilize wild type swine or bovine abattoir sources to purify the 4-hydroxyestradiol receptor away from any unique estradiol-binding protein or wild type ER protein. Subcellular fractionation studies will be done to help determine which type of receptor super family the putative methoxychlor receptor belongs. If the receptor is nuclear or cytoplasmic, and not found in the membrane, it is more likely to be a member of the steroid receptor super family. Binding affinity and specificity (competition) studies will be performed with ^{3}H-catechol estrogens synthesized and purified in our labs.

Hypothesis #3: The 4-hydroxyestradiol receptor will induce unique & specific responses distinct from both estradiol and 4-hydroxyestradiol action through ER-α and ER-β. These are best found by utilizing ovex, ER-α minus mice.

Specific Aim #3.

Messenger RNA responses to 4-hydroxyestradiol will be compared with those potentially observed with estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, and tamoxifen in the following assays:

3a. Differential display PCR in ER-α minus uteri treated with the 6 listed estrogens/compounds.

3b. Northern & *in situ* hybridization analysis for candidate mRNA responses, like progesterone receptor and glucose-6-phosphate dehydrogenase in uterus, mammary gland, and other selected tissues.

3. Rationale/Experimental Design/Methods:

Time course and dosage regimens will be as described earlier. Select mRNA candidates will be analyzed for their response to the 6 listed compounds. Candidate mRNA responses include lactoferrin, cox-1, progesterone receptor and glucose-6-phosphate dehydrogenase (51); others may be substituted depending upon the results of differential display PCR or subtractive hybridization. These mRNAs have been chosen, because they are known to respond to estradiol in uterus (2) (and therefore are estrogenic responses) and because we have shown that the first two respond to 4-hydroxyestradiol in ER-α minus uterus where estradiol does not invoke a response. Primarily we will examine the uterus and mammary gland for responses, but other tissues will be frozen away and examined, if other funding becomes available.

3. Expected Results/Potential problems:

Responses to 4-hydroxyestradiol and methoxychlor are expected. Estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, and tamoxifen are potentially responsive. From our preliminary data with the ER antagonist, ICI 182,780, it is not expected that estradiol will also work through the putative 4-hydroxyestradiol receptor. However, estradiol, methoxychlor, and tamoxifen are capable of being metabolized to catechols, which could also induce responses. We, of course, expect to see this metabolism but it should alter the same mRNAs detected

by *in situ* hybridization or DDRT-PCR as 4-hydroxyestradiol. Time course experiments and HPLC checks for metabolism of estradiol will be essential (See **Figure 1**).

Hypothesis #4: The 4-hydroxyestradiol receptor protein can be cloned by sequence homology because it is likely to be a member of the steroid receptor super family. Alternatively, the 4-hydroxyestradiol receptor cDNA can be isolated by expression cloning or sequence obtained from receptor protein affinity purification.

Specific Aim #4.

Clone the putative 4-hydroxyestradiol receptor.

4a. Test for 4-hydroxyestradiol binding and responses through Gustaffson's ER-beta.

4b. Screen for receptors that change concentration by differential display PCR analysis in the steroid receptor families using anchored oligonucleotide primers.

4c. Screen uterine ER-α minus mouse cDNA library with probes from conserved sequences of the steroid receptor super family via low stringency hybridization.

4d. Test expressed candidate orphan receptor cDNAs for binding to 4-hydroxyestradiol.

4e. Expression cloning using [^{3}H]- 4-hydroxyestradiol.

4f. Purification by affinity chromatography, then obtain partial peptide sequence for raising epitope specific antibodies or synthesizing oligonucleotide probes for screening of ER-α minus cDNA libraries.

4g. After full-length cDNA clone isolation and sequencing from one of the approaches above (a-f) we will confirm or verify identity of putative receptor by:

i. *in vitro* expression and binding to labeled 4-hydroxyestradiol, or

ii. testing for transcriptional activation of a lactoferrin promoter reporter construct (or other 4-hydroxyestradiol-responsive promoter) with the receptor bound to 4-hydroxyestradiol.

4h. Tissue specific localization of 4-hydroxyestradiol receptor mRNA expression will be ascertained by RT-PCR, Northern blot analysis, or *in situ* hybridization.

4. Rationale / Experimental Design / Methods:

To better elucidate the function of the 4-hydroxyestradiol NCER protein (putative 4-hydroxyestradiol receptor or 4OHER), we must isolate and clone its cDNA. For thoroughness we will need to check the binding of 4-hydroxyestradiol to Gustafsson's expressed ER-beta clone (22) which we have PCR amplified from mouse. 4-Hydroxyestradiol binding ability and/or transcriptional induction characterization of any NCER protein is essential for verification that we have isolated the correct receptor protein. For expression cloning, we will need a way to distinguish the NCER protein from the ER protein, hopefully by their steroid binding characteristics. Thus, detailed specificity, saturability and affinity binding studies will be needed from Specific Aims #1 and #2.

An unpublished, but highly successful method utilized by the PI in the isolation of orphan steroid receptors, is to utilize differential display PCR with anchored oligonucleotides from highly conserved regions from the gene family one is trying to clone, rather than the normal anchored oligos from the poly A tail. The twist here is to utilize DDRT-PCR rather than simple degenerate or low stringency PCR, because then one can screen only for mRNAs that change in concentration after 4-hydroxyestradiol exposure. Because, most steroid receptors down regulate their own mRNA levels, this allows one to distinguish the 4-hydroxyestradiol receptor sequence from other members of its gene family which will have very similar sequences. This is a significant problem because some gene families, like the steroid/nuclear receptor families, contain over a hundred members.

The most widely used procedure with which we have a great deal of experience is the screening of libraries with low stringency probes or PCR amplification under low stringency conditions. The probe used will be from the first zinc finger of the DNA-binding domain of the steroid receptor super family, similar to what the PI utilized in the cloning of the androgen receptor gene and cDNA (52-54).

Candidate cDNA approaches using the proteins discussed in **Footnote Table 1,** (ERR1 and ERR2) also will be tested because of their homology to the estrogen receptor. An alternative procedure to clone the putative 4-hydroxyestradiol receptor would be to prepare ER-α minus uterine cDNA expression libraries in mammalian cells and screen for [^{3}H]- 4-hydroxyestradiol -binding activity (55-57).

While affinity column purification is a potential approach, it is not being proposed as the primary approach, because of both the probable low amounts of activity and the higher efficiency of other approaches.

However, an affinity column made from a 4-hydroxyestradiol derivative would likely bind to the ER protein (14). This ER binding to the column could be blocked with estradiol and then an easily obtained abattoir source of receptor from pig or cow uterus would be used. Using this abattoir source to obtain sufficient quantities of purified protein for amino acid sequencing and/or antibody preparation will be difficult, but we do have experience in this type of purification (53). Oligonucleotides generated from protein sequence data or antibodies raised against the NCER protein (or synthetic peptide fragments) will allow cloning of the NCER cDNA from a library for further studies (52,54).

Finally, we will need to express the protein and demonstrate its 4-hydroxyestradiol-binding characteristics or transcriptional activation ability to confirm that we have cloned the correct receptor. Localization by *in situ* hybridization would confirm functional response data from Aim #1 and potentially lead to additional tissues capable of responding to 4-hydroxyestradiol.

4. Expected Results/Potential problems:

A 4-hydroxyestradiol receptor clone will be isolated that is a member of the steroid receptor super family. ER-beta is unlikely to be the 4-hydroxyestradiol receptor because ER-beta can bind and be activated by estradiol and the 4-hydroxyestradiol response was not affected by estradiol competition (preliminary data not shown). Studies of 4-hydroxyestradiol binding to ER-beta and of 4-hydroxyestradiol activation of transcription are needed to determine if ER-beta is mediating the actions of methoxychlor.

Potential problems are legion in the cloning of any novel receptor, but standard biochemical and molecular biology techniques (which the PI is familiar with) are available to surmount them. Subcellular localization will help determine if it is a nuclear receptor. However, if it should localize to the nucleus and/or happen to not be a member of the steroid receptor super family, then approaches 4e and 4f should still be viable. Prioritization of effort will be to try approaches 4a, 4b, 4c, then 4d before going to 4e. Then finally, if necessary, we will develop and utilize an affinity column approach (4f).

General Methods:

Procedures for *In Situ* Hybridization Analysis

(To study estrogen responses, we examined the cell-type specific expression of the LF gene in the mouse uterus by *in situ* hybridization (58,59) in collaboration with SK Dey and SK Das, UKMC (102). In control experiments wild type, ovariectomized mice (C57BL/6) were given a single injection (sc) of oil (0.1 ml/mouse), estradiol-17β (250 ng/mouse), kepone (15 mg/kg), the antiestrogen ICI-182,780 (50 ug/mouse) or the same dose of E_2 30 min after an injection of the same of ICI. Mice were killed 12 h after the last injection and their uteri collected for in situ hybridization.

Labeling and Purification of Catechol Estrogens

Radioactive chemicals are obtained from Dupont NEN (Boston, Massachusetts). Hexa-labeled [6,7-^{3}H]-estradiol is enzymatically treated with human cyp1b1 (4-hydroxylase) with NADPH as a cofactor (60). Since both 2- and 4-hydroxyestradiol (primarily the later) are produced in this system, labeled catechol estrogens are separated and purified by HPLC in Wade Welshons' laboratory (60). Catechol estrogens have stability problems due to oxidation and must be purified by HPLC before use (60). Stability of the labeled catechol estrogens is preserved by addition of 10 mM ascorbic acid to all buffers.

[^{3}H]-Estradiol or [^{3}H]-4-hydroxyestradiol binding assay

Sexually mature mice of each ER genotype are ovariectomized and 7 days later cytosol and nuclear extracts are prepared. Aliquots of each are assayed for [^{3}H]-estradiol or [^{3}H]-4-hydroxyestradiol binding as described previously (61). For normalization of binding data, DNA content of the nuclear fraction is measured using the procedure of Labarca and Paigen (62).

Preparation of mouse uterine cells for primary cell culture.

Immature or ovariectomized female ER-α minus or wild type mice are utilized in the isolation of uterine cells (24). Estrogen-free growth medium is replaced with fresh growth medium the day after plating. Medium is changed again the two days prior to experimentation.

[^{3}H]-Estradiol and [^{3}H]-4-hydroxyestradiol binding - Whole cell uptake assay

This binding assay is performed according to the method of Welshons *et al.* (41,63). Cells will be incubated at 37°C in medium labeled with [^{3}H]estradiol or [^{3}H]-4-hydroxyestradiol in the presence (nonspecific binding) or absence (total binding) of a 100-fold excess of unlabelled ligand. For normalization of binding data, well content of DNA and protein are measured, using the procedures of Labarca and Paigen (62) and Bradford (64) respectively.

Differential display reverse transcriptase PCR (DDRT-PCR) analysis of mRNA

DDRT-PCR is a powerful tool for the analysis of subtle changes in gene expression in tissues and cell lines (65). Early use of this technique has had mixed success in laboratories around the world, but with improvements in methods it has become more reliable (66). This technique allows detection of differentially expressed genes without using specific probes for known gene products and is ideally suited to our purposes of detecting potential subtle 4-hydroxyestradiol, methoxychlor or estradiol, as well as other estrogens, responses in ER-α minus mice. RNA is isolated using a kit (Purescript RNA Isolation Kit) obtained from Gentra Systems, Inc. (Minneapolis, MN). Differential display of mRNA is examined using a kit obtained from GenHunter Corporation (Brookline, MA) (65-67). Steroid-regulated gene expression can ideally be studied by DDRT-PCR because there are relatively few changes in gene expression that occur. The PI has in the past successfully used the technique with "diabetic" tissue culture cell models to isolate novel glucose-responsive orphan steroid receptors from diabetic model cell lines (data not shown, 68). The method is more reproducible in *vitro* culture than *in vivo,* but with three separate uteri, each being run in duplicate, (6 samples per treatment) we reduce our problems with false positives. However, differentially expressed mRNA bands, which are isolated, cloned into plasmids, and expression changes must be confirmed by analysis as probes on Northern gels, by *in situ* hybridization, or by quantitative PCR. After confirmation and sequencing, these probes are used as markers of differential gene expression due to the presence or absence of ER or a particular estrogen treatment.

Quantitation of LF mRNA levels by RT/competitive polymerase chain reaction of the Effects of Estradiol, Kepone or 4-Hydroxyestradiol on Uterine Lactoferrin mRNA levels in Ovariectomized Wild Type or ER-α Minus Mice

Construction of the mutant templates.

To perform a competitive PCR for LF mRNAs, a mutant template (the competitor), containing the same primer template sequences as those of target cDNA competing for primer annealing and amplification, is generated by introducing a non-specific DNA fragment into a mouse LF cDNA clone (71). A 185 bp blunt-ended fragment (SspI) obtained from pGEM7Zf(+) vector, was ligated with the LF cDNA in pGEM4Z at the StuI site. This DNA construct was used to serve as a competitor template to carry out the quantitation of LF mRNA levels in uterine tissues.

RT and competitive PCR.

Total RNA (1 μg) was reverse-transcribed using an antisense oligo (5' GGAACACAGCTCTTTGAGAAGAAC 3') for mouse LF mRNA. The protocol for the RT reaction, for the PCR reaction and the cycle parameters have been described (72). The competitive PCR was performed using the method as described (73). In brief, a fixed amount (1/10th) of the total RT product and increasing amounts (10-fold serial increases) of the mutant template are co-amplified for 30 cycles by PCR, using the mixture of sense (5' AGGAAAGCCCCCCTACAAAC 3') and antisense (as shown above) oligos. The PCR amplified products were analyzed by Southern hybridization using a ^{32}P-end labeled internal oligo (5' CTGCTGTTCTTCACGACTGCTACC 3'). Direct radioimaging of the Southern blot was performed by Ambis

image analysis system to estimate the radioactive intensity of the bands of the target cDNA (276 bp) and competitor cDNA (461 bp). The ratio of band radioactive intensities of the competitor and target cDNAs was calculated for each sample and plotted against the amounts of competitor. The amount of target cDNA is determined from the logarithm plot at zero equivalence point. The efficiency of RT reaction was controlled by measuring the ribosomal protein L-7 (rpl7) mRNAs levels in each sample.

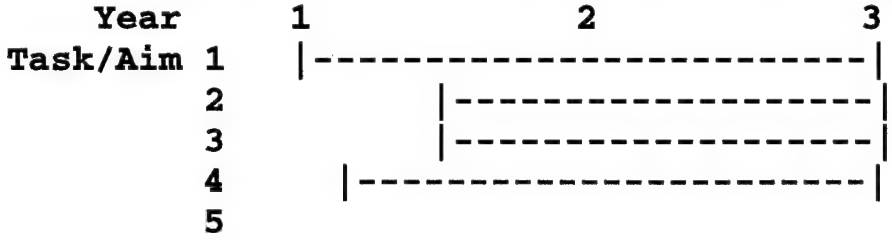
Molecular Biology / Genetic Procedures

Standard procedures will be utilized unless specifically modified as indicated (69,70).

Statement of Work:

New Timetable for the proposed experiments:

The diagram below outlines likely emphasis over the remaining years of support.



Progress on Tasks:

Task/Specific Aim #1. Characterize lactoferrin mRNA responses to 4-hydroxyestradiol in ER-α minus mice. Specificity and dose response.

Ongoing. A paper has been published describing the specificity of lactoferrin mRNA response to 4OHE2 (102, See Appendix). We have had a surprisingly hard time getting RNA from mouse tissues uncontaminated by lactoferrin amplified DNA so that we can do the dose response for 4-hydroxyestradiol. It should be possible because we were able to get a nice saturable dose response curve for methoxychlor which may be acting through a catechol mechanism as well (103). We are likely to replace the in situ approach with a Taq Man quantitation approach because of the ease of analysis and the more quantitative nature of this new technique.

Task/Specific Aim #2. Characterize the putative 4-hydroxyestradiol receptor in ER-α minus mice. Subcellular fractionation and dose response for some ^{3}H-4-hydroxyestradiol.

Ongoing. We are about to label up some ^{3}H-4-hydroxyestradiol made from ^{3}H-estradiol catalyzed by human 4-hydroxylase obtained from either Colin Jefcoate or GenTest, Inc (Woburn, Mass.). This will provide the reagents needed for these *in vitro* studies.

Task/Specific Aim #3. Compare the specificity of the responses to 4-hydroxyestradiol with those of estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, and tamoxifen in ER-α minus mice. Differential display and candidate responses.

Ongoing. Early results by differential display PCR are promising for a specific mRNA response unique to 4-hydroxyestradiol but not inhibited by ICI or observed with estradiol, thus indicating the 4OHE2 response is not mediated by ER-α or ER-β. Based upon elevated uterine lactoferrin mRNA expression by 4-hydroxyestradiol in ovariectomized ERαKO female mice via a non-ERα, non-ERβ mechanism (102), we employed differential display analysis to identify other estrogen-responsive genes uniquely regulated by 4-hydroxyestradiol in the uteri ERαKO female mice.

In this task, the differential display technique identified a putative down-regulated gene in response to 4-hydroxyestradiol, but not to 17β-estradiol, in the uteri of ovariectomized ERαKO mice. An estrogen receptor antagonist, ICI 182,780, failed to inhibit this down-regulation induced by 4-hydroxyestradiol. Currently, the

cDNA product is being further amplified and will subsequently be purified and sequenced. Differential display RT-PCR identified a potential gene product whose expression is down regulated by 4-hydroxyestradiol, but not by 17β-estradiol, in ERαKO mouse uterine tissue (**See Figure 2 below**). The failure of ICI 182,780 to antagonize gene down-regulation suggests a non-ERα, non-ERβ mechanism for 4-hydroxyestradiol-induced uterine gene expression. The ability of 4-hydroxyestradiol to down-regulate gene expression independent of ERα/ERβ supports the hypothesis that catechol estrogens are biologically active compounds in their own right and not merely benign excretory products

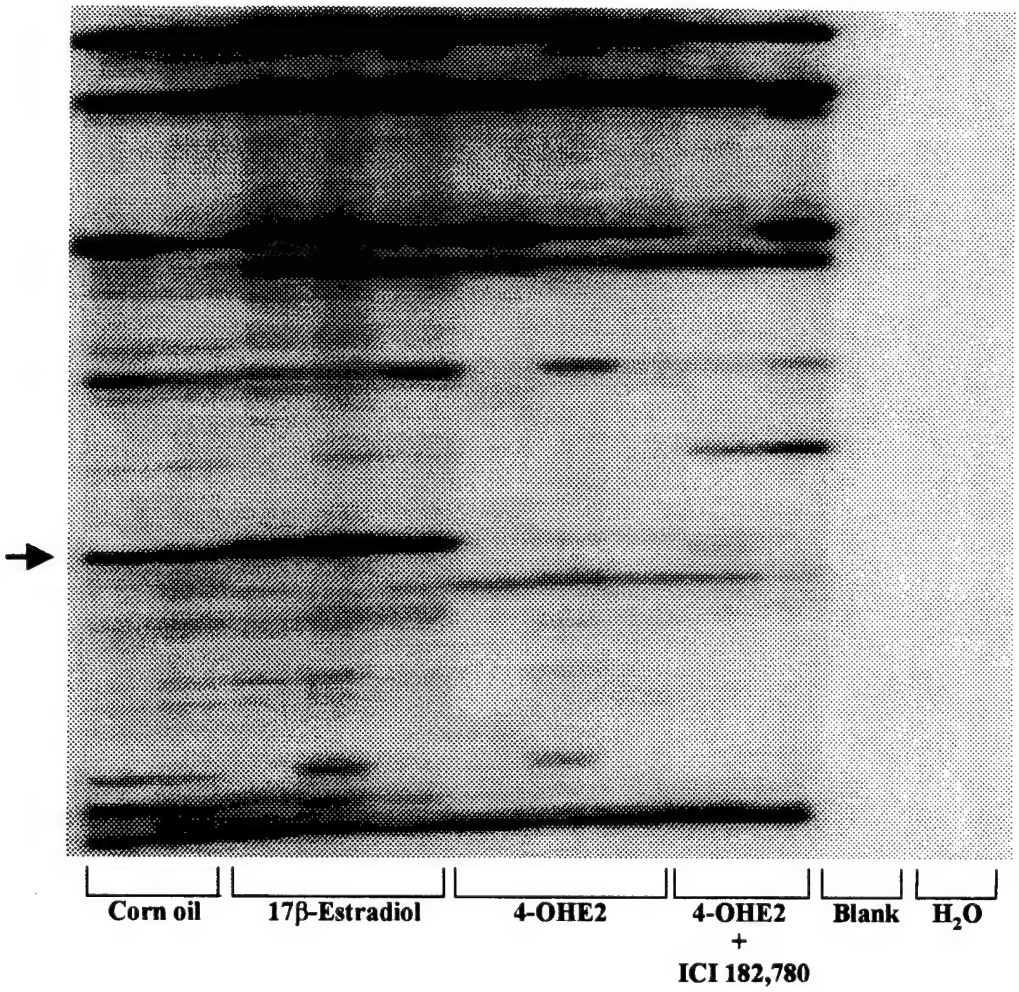


Figure 2. Differential display of uterine mRNAs from adult homozygous (-/-) ERαKO mice. Animals were ovariectomized and given two i.p. injections of corn oil, 17β-estradiol, 4-hydroxyestradiol or 4-hydroxyestradiol + ICI 182,780. Mice were sacrificed 12 hours after the second treatment and total uterine RNA was isolated and used to provide mRNA for differential display. PCR products were analyzed on a 6% polyacrylamide gel and visualized by autoradiography. Arrow indicates amplified cDNA product down-regulated by 4-hydroxyestradiol in ERαKO mouse uterine tissue.

Task/Specific Aim #4. Clone the putative 4-hydroxyestradiol receptor (4OHER).

4a. Test for 4-hydroxyestradiol binding and responses through Gustaffson's ER-beta.
4b. Screen for receptors that change concentration by differential display PCR analysis in the steroid receptor families using anchored oligonucleotide primers.
4c. Screen uterine ER-α minus mouse cDNA library with probes from conserved sequences of the steroid receptor super family via low stringency hybridization.
4d. Test expressed candidate orphan receptor cDNAs for binding to 4-hydroxyestradiol.
4e. Expression cloning using [^{3}H]- 4-hydroxyestradiol.
4f. Purification by affinity chromatography, then obtain partial peptide sequence for raising epitope specific antibodies or synthesizing oligonucleotide probes for screening of ER-α minus cDNA libraries.
4g. After full-length cDNA clone isolation and sequencing from one of the approaches above (a-f) we will confirm or verify identity of putative receptor by:
i. *in vitro* expression and binding to labeled 4-hydroxyestradiol, or
ii. testing for transcriptional activation of a lactoferrin promoter reporter construct (or other 4-hydroxyestradiol-responsive promoter) with the receptor bound to 4-hydroxyestradiol.
4h. Tissue specific localization of 4-hydroxyestradiol receptor mRNA expression will be ascertained by RT-PCR, Northern blot analysis, or *in situ* hybridization.

Progress:

Ongoing.

4a. 4-hydroxy estradiol has been shown in a paper by Gustafsson in March 1997 Endocrinology to bind to both ER-α and ER-β. However, this is not the mechanism of the 4-hydroxy estradiol response we have been observing because ER-β is blocked/binds by ICI and estradiol. We are currently testing a new hypothesis that an alternatively spliced form of ER-α or ER-β and/or a heterodimerization of the two may provide unique specificity for 4-hydroxy estradiol.

4b-4f. Ongoing but very early stages. No results yet.

Conclusions:

Endogenous estrogens can be hydroxylated at multiple sites by NADPH-dependent cytochrome P450 enzymes. The catechol estrogens, 2- and 4-hydroxyestradiol, are a major group of estrogen metabolites formed by the aromatic hydroxylation of 17β-estradiol at the C-2 and C-4 positions, respectively. In mammalian species, catechol estrogen formation from 17β-estradiol is quantitatively the most important metabolic pathway of this endogenous sex hormone. Among the different metabolites of 17β-estradiol, only 2- and 4-hydroxyestradiol have been found to bind to both ERα and ERβ with a relatively high affinity. Although previously believed to be to be benign excretory products, recent evidence suggests that catechol estrogens may be local mediators of estrogen action that possess potent biological and endocrine activities of their own (102,103). In MCF-7 cells, 2- and 4-hydroxyestradiol have been shown to significantly stimulate cell growth and increase progesterone receptor. Effects on embryo implantation, gonadotropin release, partruition and increases in uterine weight also have been reported. More importantly, catechol estrogens have been implicated in hormone-induced carcinogenesis as reviewed earlier (74-101).

To better understand the receptor mechanisms mediating the multitude of estrogenic effects, Lubahn *et al.* (1) generated ERα "knock-out" (ERαKO) mice using homologous recombination techniques. In these transgenic mice, uterine mRNA expression of the estrogen-responsive gene lactoferrin has been shown to be up-regulated by the catechol estrogen 4-hydroxyestradiol and methoxychlor, but not by 17β-estradiol (102, 103). These results suggest the presence of a novel non-ERα, non-ERβ estrogen signaling pathway. We have identified via differential display RT-PCR an additional potential gene whose expression is uniquely down regulated by the catechol estrogen 4-hydroxyestradiol in the uteri of ovariectomized ERαKO mice.

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Biochemistry

Estrogenic responses in estrogen receptor-α deficient mice reveal a distinct estrogen signaling pathway

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ABSTRACT **Estrogens are thought to regulate female reproductive functions by altering gene transcription in target organs primarily via the nuclear estrogen receptor-α (ER-α). By using ER-α "knock-out" (ERKO) mice, we demonstrate herein that a catecholestrogen, 4-hydroxyestradiol-17β (4-OH-E_2), and an environmental estrogen, chlordecone (kepone), up-regulate the uterine expression of an estrogen-responsive gene, lactoferrin (LF), independent of ER-α. A primary estrogen, estradiol-17β (E_2), did not induce this LF response. An estrogen receptor antagonist, ICI-182,780, or E_2 failed to inhibit uterine LF gene expression induced by 4-OH-E_2 or kepone in ERKO mice, which suggests that this estrogen signaling pathway is independent of both ER-α and the recently cloned ER-β. 4-OH-E_2, but not E_2, also stimulated increases in uterine water imbibition and macromolecule uptake in ovariectomized ERKO mice. The results strongly imply the presence of a distinct estrogen-signaling pathway in the mouse uterus that mediates the effects of both physiological and environmental estrogens. This estrogen response pathway will have profound implications for our understanding of the physiology and pathophysiology of female sex steroid hormone actions in target organs.**

The precise mechanism(s) of action of a number of estrogenic compounds are as yet poorly understood. Traditionally, the consensus has been that all estrogens act via the classical estrogen receptor-α (ER-α), but the recent identification of another form of ER, ER-β, in mammalian tissues including uterus and ovary (1–5) has necessitated a reevaluation of the actions of estrogenic compounds in target organs. The relative distribution and levels of ER-α and ER-β vary considerably, but the relative biological significance of these two receptor forms is not yet known.

It was recently reported that natural estrogens, catecholestrogens and xenoestrogens (traditionally thought to act via ER-α) bind to both ER-α and ER-β (6). These compounds have estrogenic activities, although their specific action mechanisms remain poorly understood. Catecholestrogens, 2-hydroxyestradiol-17β (2-OH-E_2) and 4-hydroxyestradiol-17β (4-OH-E_2), are produced from primary estrogens via hydroxylation at the C2 or C4 position respectively by catecholestrogen synthases (7). These two estrogens possess differential physiologic potencies and functions: for example, the uterotrophic potency of 4-OH-E_2 is close to that of estradiol-17β (E_2), whereas 2-OH-E_2 is considerably weaker (7, 8). Although catecholestrogens have similar binding affinities for ER-α and ER-β, these binding affinities are about 7- to 14-fold lower than that of the primary estrogen, E_2 (6). Xenoestrogens are environmental estrogens, drawing increasing attention today for their potential adverse effects on both human and animal reproduction (9–11). Their reproductive toxicity is widely believed to be mediated by their binding to ER-α and mimicking certain effects of primary estrogens (12), but again, the specific mechanisms are ill-defined.

The ER-α knockout (ERKO) mouse (13) does not respond to E_2 with respect to uterine expression of lactoferrin (LF), progesterone receptor or glucose-6-phosphate dehydrogenase genes, all of which are E_2-responsive in the wild-type uterus (14, 15). This lack of responsiveness to E_2 is observed even though uteri of ovariectomized ERKO mice still show ≈5–10% of the E_2 binding exhibited in wild-type uteri (13). In wild-type mice, E_2 can modulate the expression of a subset of uterine genes that are likely to alter cellular responses (15), but whether catecholestrogens or xenoestrogens can alter the expression of these same genes in a manner similar to that of E_2 is not known. In this study we explore the ER-α vs. non-ER-α-mediated actions of 4-OH-E_2 and kepone (a xenoestrogen), and examine estrogen-induced gene responses in the ERKO mouse.

MATERIALS AND METHODS

Animals and Injections Schedule. Adult wild-type (+/+) or homozygous (−/−) ERKO sibling mice of the same genetic background (129/J/C57BL/6J) were ovariectomized and rested for 2 weeks before treatment. All treatments were given as two injections at 6-h intervals of oil (control), kepone (15 mg/kg body weight), E_2 (10 μg/kg), 4-OH-E_2 (10 μg/kg), or ICI-182,780 (ICI, 1 or 20 mg/kg). In addition, the following combinations of treatments (at the same doses) were administered: ICI 30 min before the injections of E_2, 4-OH-E_2 or kepone, or E_2 30 min before the injections of 4-OH-E_2. Mice were killed 6 h after the last injection. All of the test agents were dissolved in corn oil and injected (0.1 ml/mouse) subcutaneously.

***In Situ* Hybridization.** *In situ* hybridization was performed as described (15). Each uterine horn was excised, cut into halves, and flash-frozen in freon. Frozen sections (10 μm) were mounted onto poly-L-lysine coated slides and fixed in 4% paraformaldehyde in PBS for 15 min at 4°C. Following prehybridization, uterine sections were hybridized with ^{35}S-labeled LF sense or antisense cRNA probes for 4 h at 45°C. After hybridization and washing, the sections were incubated with RNase-A (20 μg/ml) at 37°C for 15 min. RNase-A resistant hybrids were detected by autoradiography after 3–5 days of exposure using Kodak NTB-2 liquid emulsion. The slides were poststained with hematoxylin and eosin.

Northern Blot Hybridization. For Northern blot hybridization, total uterine RNA (6.0 μg) was denatured and separated

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Abbreviations: ER, estrogen receptor; ERKO, ER-α knock out; 4-OH-E_2, 4-hydroxyestradiol-17β; LF, lactoferrin; rpL7, ribosomal protein L-7; ICI, ICI-182,780; E_2, estradiol-17β.

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by formaldehyde/agarose gel electrophoresis, transferred to nylon membranes, and UV cross-linked. Northern blots were prehybridized, hybridized, and washed as described (15). Hybridization was performed in sequence with ^{32}P-labeled cRNA probes (specific activity, $\approx 2 \times 10^9$ dpm/μg) for mouse LF and ribosomal protein L-7 (rpL7). Transcripts were detected by autoradiography (2-h exposure time). Quantitation of radioactivity in hybridized bands was achieved by radioimage analysis (Ambis Systems, San Diego).

Competitive PCR. The method of quantitation of mRNAs by competitive PCR is described elsewhere (16) and was chosen because of the limited amount of RNA obtainable from ERKO mouse uteri. In brief, the competitor template containing the same primer template sequences as those of the target cDNA competing for the primer annealing and amplification was generated by introducing a nonspecific DNA fragment into a mouse target cDNA clone. Specifically, a 185-bp blunt-ended fragment (*Ssp*I), obtained from pGEM7Zf(+) vector, was inserted into the LF cDNA at the *Stu*I site or into the rpL7 cDNA at *Bgl*II site. These DNA templates were used as competitors to carry out the competitive PCR for LF and rpL7 cDNA templates derived by reverse transcription (RT) reaction in uterine RNAs. For RT-PCR the following primers were used: 5′-AGGAAAGCCCCCCTACAAAC-3′ (nt 258–277, sense), 5′-GGAACACAGCTCTTTGAGAAGAAC-3′ (nt 510–533, antisense) for LF mRNA (17), and 5′-TCAATGGAGTAAGCCCAAAG-3′ (nt 359–378, sense) and 5′-CAAGAGACCGAGCAATCAAG-3′ (nt 585–604, antisense) for rpL7 RNA (18). The internal oligonucleotides, 5′-CTGCTGTTCTTCACGACTGCTACC-3′ (nt 339–362, antisense) and 5′-GATTGCCTTGACAGATAATTC-3′ (nt 564–584, sense) were used for Southern blot hybridization of RT-PCR amplified products for LF and rpL7, respectively. Protocols for the RT reaction and PCR amplification were as described (19). In brief, one-tenth of total RT reaction product was coamplified with 10-fold increasing amounts of the competitive template (1–100,000 fg for wild-type or 1–10,000 fg for ERKO samples) by PCR for 30 cycles, with the mixture of sense and antisense oligonucleotides. Amplified products were separated by 1% agarose gel electrophoresis and detected by Southern blot hybridization by using a ^{32}P-labeled internal oligonucleotide. Quantitation of radioactivity in hybridized bands was achieved by radioimage analysis (Ambis Systems). The ratio of radioactivities of the competitor and target cDNA was calculated for each sample and plotted against the amounts of competitor. The amount of target cDNA was determined from the logarithm plot at zero equivalence point, which represents 10% of the total; i.e., only one-tenth of total reaction was used.

Effects of E_2 or 4-OH-E_2 on Uterine Phase I Estrogenic Responses in ERKO Mice. Phase I responses were evaluated

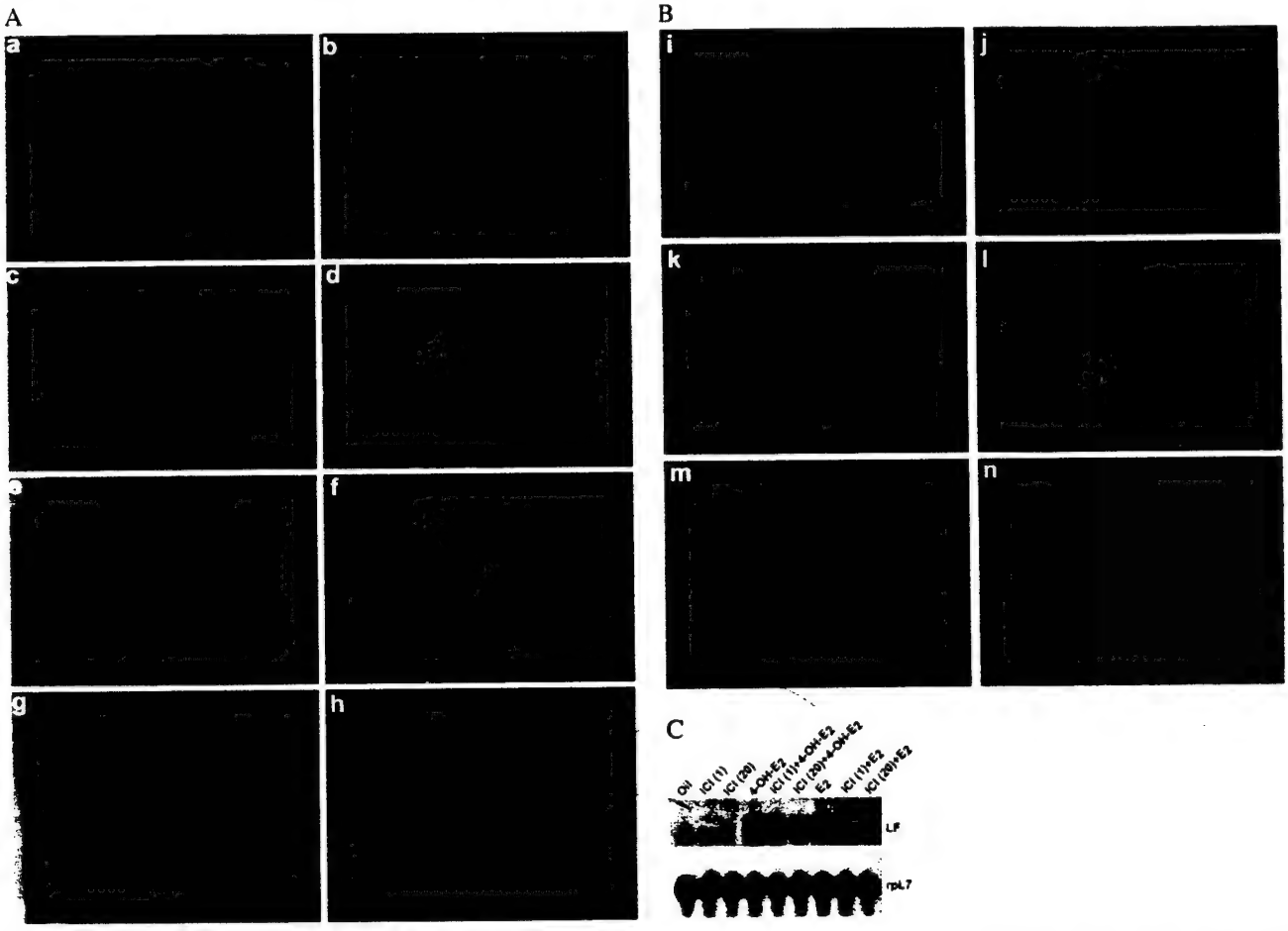

FIG. 1. *In situ* hybridization of LF mRNA in the ovariectomized wild-type mouse uterus (*A*) after exposure to oil (control), E_2 (10 μg/kg), and/or ICI (20 mg/kg), and (*B*) after exposure to 4-OH-E_2 (10 μg/kg) and 4-OH-E_2 plus ICI or kepone (15 mg/kg). Bright- and dark-field photomicrographs of uterine sections are shown. (×100.) (*A a* and *b*) Oil (vehicle). (*c* and *d*) E_2. (*e* and *f*) ICI + E_2. (*g* and *h*) ICI. (*B i* and *j*) 4-OH-E_2. (*k* and *l*) ICI + 4-OH-E_2. (*m* and *n*) Kepone. No positive signals were observed when sections were hybridized with the sense probe (data not shown). LE, luminal epithelium; GE, glandular epithelium; S, stroma. These experiments were repeated three times with three mice in each group, and similar results were obtained. (*C*) Northern blot analysis of LF and rpL7 mRNAs. Effects of ICI {1 mg/kg [ICI (1)] or 20 mg/kg [ICI (20)]} on E_2- or 4-OH-E_2-induced uterine LF mRNA levels in wild-type mice are shown. The pooled total RNA (6 μg) obtained from three to four mice in each group was analyzed. These experiments were repeated twice with independent RNA samples and similar results were obtained.

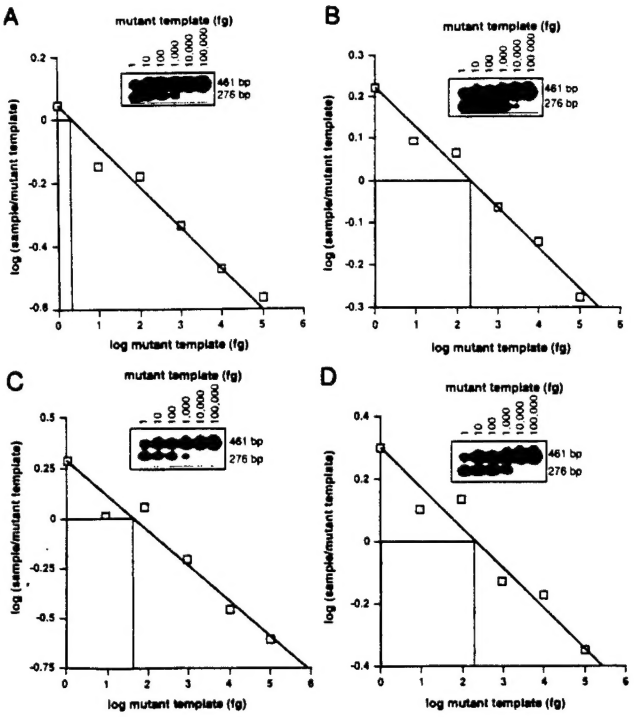

FIG. 2. Competitive RT-PCR for LF mRNA in ovariectomized wild-type mouse uterus. Adult ovariectomized wild-type mice were injected with oil (*A*), E_2 (*B*), kepone (*C*), or 4-OH-E_2 (*D*). The pooled uterine total RNA (1 μg) obtained from three to four mice in each group was reverse-transcribed by using LF or rpL7 mRNA specific antisense oligonucleotides as described. Two products of 461 and 276 bp for the competitor and sample DNA templates, respectively, were detected for LF mRNA. The ratios of radioactivities of the sample bands to those of the mutant are plotted against the amounts of the competitor template. The efficiency of the RT reaction was controlled by measuring the level of rpL7 mRNA in each sample, which was similar in all samples (4.0×10^7 copies/μg of total RNA). These experiments were repeated twice with two independent sets of RNA samples, and similar results were obtained.

by measuring uterine water imbibition and uterine uptake of ^{125}I-labeled BSA as described (20). Ovariectomized mice were given two injections at 6-h intervals of oil (control), E_2 (10 μg/kg), or 4-OH-E_2 (10 μg/kg). At 6 h after the second injection, they were given an intravenous injection of ^{125}I-labeled BSA (0.5 μCi, specific activity 10 μCi/μg; 1 Ci = 37 GBq). After 15 min the mice were perfused with saline and killed. Uterine wet weights were recorded, followed by counting of uterine radioactivity in a γ-counter. After recording uterine radioactivity, the uterine dry weights were

Table 1. Effects of E_2, 4-OH-E_2, or kepone on uterine LF mRNA levels in ovariectomized wild-type mice

Treatments	Levels of mRNA, fg/μg total RNA	mRNA copies, molecules/μg total RNA	Fold increase
Oil	21.8	4,800	1.0
E_2	2126.4	470,000	97.6
Kepone	426.8	94,000	19.6
4-OH-E_2	1857.4	400,000	85.2

Mice were given two injections at 6-h intervals of oil (control, 0.1 ml/mouse), E_2 (10 μg/kg), 4-OH-E_2 (10 μg/kg), or kepone (15 mg/kg), and were killed 6 h after the last injection. In each treatment group the pooled uterine total RNA from three to four mice was subjected to RT-PCR as described. Values were derived from the zero equivalence point of the logarithmic plot as shown in Fig. 2, and fold increases were calculated with respect to oil value. Values represent the average of two independent experiments.

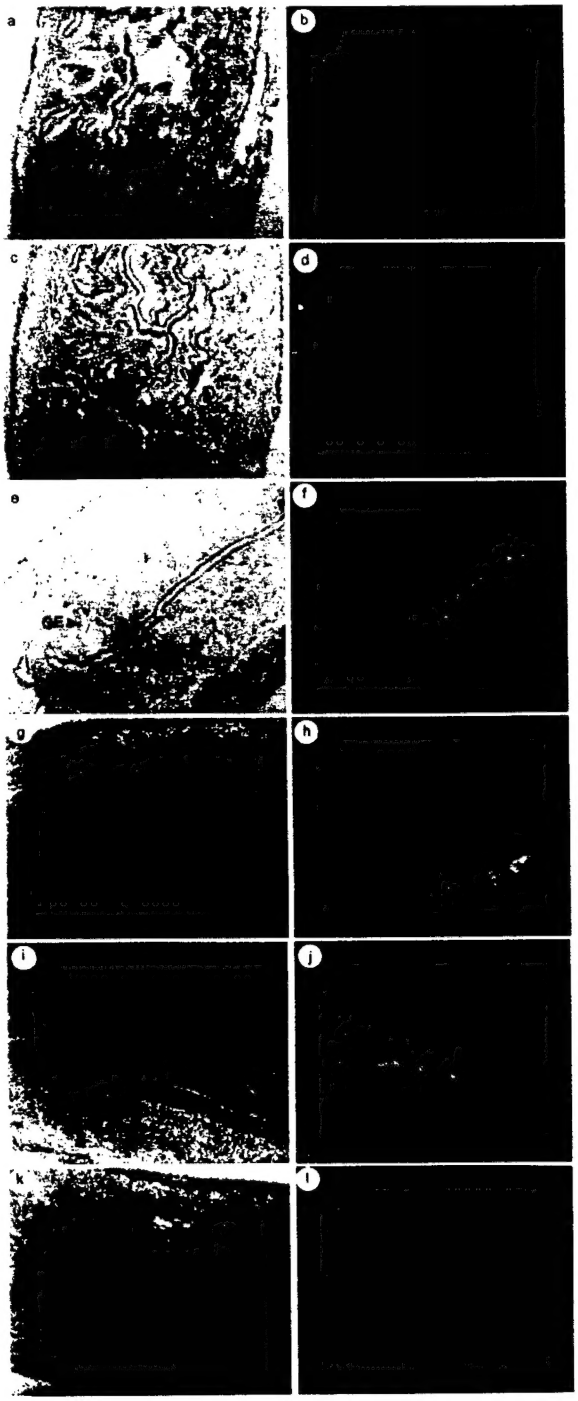

FIG. 3. *In situ* hybridization of LF mRNA in uteri of ovariectomized ERKO mice after injection of oil, E_2 (10 μg/kg), kepone (15 mg/kg), 4-OH-E_2 (10 μg/kg), 4-OH-E_2 plus ICI (20 mg/kg), or ICI alone. Bright- and dark-field photomicrographs of uterine sections are shown. (×100.) (*a* and *b*) Oil. (*c* and *d*) E_2. (*e* and *f*) Kepone. (*g* and *h*) 4-OH-E_2. (*i* and *j*) ICI + 4-OH-E_2. (*k* and *l*) ICI. Sections hybridized with the sense probe did not exhibit any positive signals (data not shown). LE, luminal epithelium, GE, glandular epithelium; S, stroma, CM, circular muscle, LM, longitudinal muscle. These experiments were repeated three times with three mice in each group and similar results were obtained.

determined, and water imbibition was calculated (wet weight minus dry weight). The specificity of uterine uptake of ^{125}I-labeled BSA was determined by comparison with uptakes in other tissues, such as skeletal muscle, liver, and heart.

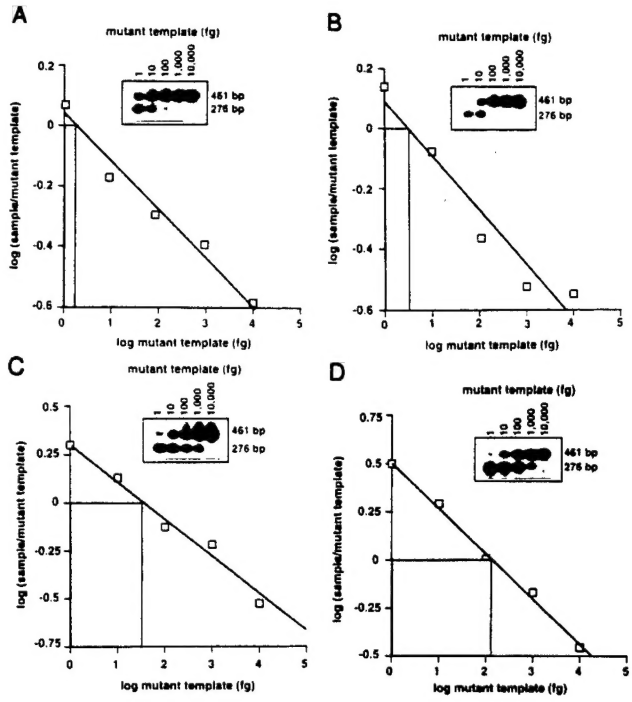

FIG. 4. Competitive RT-PCR for LF mRNA in the ovariectomized ERKO mouse uterus. Adult ovariectomized ERKO mice were injected with oil (*A*), E_2 (*B*), kepone (*C*), or 4-OH-E_2 (*D*) as described in Fig. 2. The pooled uterine total RNA (1 μg) obtained from three to four mice in each group was reverse-transcribed by using LF or rpL7 mRNA specific antisense oligonucleotides as described. These experiments were repeated at least four times with independent RNA pools and similar results were obtained.

Statistics. Statistical analyses were performed by using one-way ANOVA followed by Newman–Keul's multiple range test and by Student's *t* test.

RESULTS AND DISCUSSION

LF is a major estrogen-induced secretory iron-binding glycoprotein of the mouse uterine epithelium, and is thought to have bactericidal and growth-promoting properties (17). E_2-induced activation of the LF gene has been shown to be mediated through ER-α by an imperfect palindromic estrogen response element in the 5′-flanking region of the LF gene (17). To examine whether uterine responses to 4-OH-E_2 or kepone differed from those to E_2, we compared the effects of these agents on uterine LF mRNA levels in ovariectomized ERKO and wild-type mice by using *in situ* hybridization, Northern blot analysis and quantitative PCR.

The results of *in situ* hybridization showed that 4-OH-E_2 and kepone, like E_2, up-regulated the LF mRNA accumulation in wild-type uterine epithelial cells (Fig. 1*A* and *B*). The up-regulation by E_2 was dramatically reduced by pretreatment with the pure antiestrogen ICI at 20 mg/kg (Fig. 1*A*). In contrast, the up-regulation by 4-OH-E_2 was only partially inhibited by this antiestrogen (Fig. 1*B*). These results were further confirmed by Northern blot hybridization (Fig. 1*C*). Thus, 16-fold induction of uterine LF mRNA levels by E_2 was remarkably reduced to only 1-fold by pretreatment with ICI at 20 mg/kg. Quantitation by radioimage analysis revealed that the inhibitory effects of ICI were dose-dependent. In contrast, 15-fold induction by 4-OH-E_2 was only reduced to 11-fold by pretreatment with ICI at 20 mg/kg. The antiestrogen alone did not influence the expression of uterine LF mRNA (Fig. 1*A* and *C*). By using a different methodology, quantitative RT-PCR demonstrated ≈98-, 85-, and 20-fold induction of LF mRNA in the wild-type uterus by E_2, 4-OH-E_2, and kepone, respectively (Fig. 2 and Table 1). Collectively, these results indicate that under normal conditions the uterus responds to these three estrogenic compounds with respect to LF gene expression. Moreover, only partial inhibition of 4-OH-E_2-induced uterine LF mRNA accumulation by ICI suggested that catecholestrogens and presumably xenoestrogens are coupled to an additional and independent signaling pathway not involving ER-α. This speculation was tested by using ovariectomized ERKO mice.

As reported previously (14), E_2 was ineffective at inducing LF mRNA in uterine epithelial cells of ERKO mice (Fig. 3). Surprisingly, both 4-OH-E_2 and kepone stimulated LF mRNA levels in these cells (Fig. 3) with induction levels of ≈60- and 19-fold for 4-OH-E_2 and kepone, respectively (Fig. 4 and Table 2). This induction of uterine LF mRNA by 4-OH-E_2 or kepone was not suppressed by pretreatment with E_2 or ICI (Table 2). It is also interesting to note that 4-OH-E_2 showed 25-fold greater induction of LF mRNA levels in wild-type mice than in ERKO mice (Table 1 vs. Table 2), suggesting that in wild-type mice, 4-OH-E_2 can affect the expression of the LF gene via both ER-α and non-ER-α mediated pathways.

Several primary estrogens are known to exert both early (phase I) and late (phase II) responses in the uterus (20). As shown in Fig. 5, the treatment of ERKO mice with 4-OH-E_2 significantly increased two phase I estrogenic responses, uterine water imbibition, and ^{125}I-labeled BSA uptake. In contrast, E_2 failed to elicit any significant changes in these phase I responses (Fig. 5). No significant changes in ^{125}I-labeled BSA

Table 2. Levels of uterine LF mRNA in ovariectomized ERKO mice after treatment with various agents

Treatments	Levels of mRNA, fg/μg total RNA	mRNA copies, molecules/μg total RNA	Fold increase
Oil (n = 4)	18.9 ± 1.0[a]	4,100 ± 200[a]	1.0
E_2 (n = 4)	27.0 ± 2.7[a]	5,900 ± 200[a]	1.4
4-OH-E_2 (n = 5)	1126.5 ± 40.2[b]	240,000 ± 10,000[b]	59.6
4-OH-E_2 + ICI (n = 3)	1249.7 ± 40.5[b]	280,000 ± 10,000[b]	66.0
4-OH-E_2 + E_2 (n = 3)	1152.0 ± 65.9[b]	250,000 ± 10,000[b]	60.9
Kepone (n = 4)	360.3 ± 24.0[c]	84,000 ± 3,000[c]	19.0
Kepone + ICI (n = 3)	369.7 ± 22.8[c]	82,000 ± 2,000[c]	19.5

Mice were given two injections at 6-h intervals of oil (control, 0.1 ml/mouse), E_2 (10 μg/kg), 4-OH-E_2 (10 μg/kg), kepone (15 mg/kg), 4-OH-E_2 + ICI (20 mg/kg), 4-OH-E_2 + E_2, or kepone + ICI. Mice were killed 6 h after the last injection. "n" denotes the number of replicates. For each replicate the pooled uterine total RNA from three to four mice was subjected to RT-PCR as described. Values were derived from the zero equivalence point of the logarithmic plot as shown in Fig. 4, and fold increases were calculated with respect to oil value. Values with different superscript letters are statistically different ($p <$ 0.05, ANOVA followed by Newman–Keul's multiple range test).

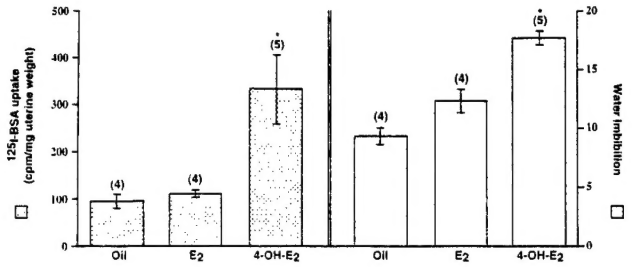

FIG. 5. Effects of E_2 or 4-OH-E_2 on uterine water imbibition (mg) and ^{125}I-labeled BSA uptake in ERKO mice treated with oil (control), E_2 (10 μg/kg), or 4-OH-E_2 (10 μg/kg). Results shown are mean ± SEM. Numbers within parentheses indicate the number of mice used for each treatment group. Values are statistically different ($P < 0.05$, Student's t test) from those treated with oil or E_2.

accumulation were noted in skeletal muscle, liver, and heart by either 4-OH-E_2 or E_2 treatment (data not shown).

Taken together, these uterine responses to estrogens in ERKO mice clearly establish the presence of a pathway that is not mediated via the classical ER-α, but could be mediated by non-ER-α estrogen signaling pathways. Interestingly, several E_2 responses have been observed in mammals lacking ER-α and are proposed to be mediated via ER-β. These mammalian E_2 responses include a small increase in ERKO mouse uterine wet weight after 10 days of high dose E_2 treatment (21), E_2 inhibition of a vascular injury response in ERKO mice (22), and sublingual E_2-induced brachial artery dilation in an ER-α negative man (23).

Although the molecular mechanism(s) by which 4-OH-E_2 or kepone alter uterine functions has yet to be definitively elucidated, several possibilities can be ruled out. First, ERKO mice may have alternatively spliced forms of ER-α (14). The existence of alternatively spliced forms of ER-α that contain the ER binding domain has recently been documented, either with sequence changes upstream of the exon 5/6 boundary in rat pituitary, or lacking exon 5 in rat brain and human smooth muscle cells (24–26). Second, it is possible that differential effects of the various "estrogenic" ligands in ERKO mice could be mediated by different ER subtypes, such as ER-β or its alternatively spliced forms.[¶] However, it is known that E_2 and antiestrogens bind to both ER-α and ER-β ligand-binding domains (6). Our observations demonstrate that E_2 is unable to influence ERKO uterine LF expression, and that neither E_2 nor ICI inhibits 4-OH-E_2- or kepone-induced uterine LF expression in ERKO mice. This lack of inhibition suggests that the effects of this catecholestrogen and xenoestrogen on the uterine LF gene are not mediated via the ligand binding domains of ER-α or ER-β. Further, the level of ER-β is remarkably low in comparison to ER-α in the wild-type mouse uterus (data not shown), and is very low to absent in ERKO mouse uterus.[‖]

Alternative mechanisms for the mediation of estrogenic responses that cannot be described in terms of either ER-α or ER-β could be via membrane estrogen receptors (27, 28) or other response proteins, such as a novel orphan receptor member of the nuclear receptor superfamily (29). It should also be noted that response to an "estrogen" in a target tissue is not necessarily related to its affinity for or its occupancy of the receptor (30–32), and estrogen-responsive genes with no recognizable estrogen response element have been identified (33–35). In addition, it is now apparent that at least 10 coactivator proteins or basal factors form combinatorial complexes that modulate receptor function (36–39), and ER-α and ER-β themselves form a heterodimeric complex (40, 41). All of these ER complexes have the potential to differentially affect the regulation of gene expression by estrogens or even the receptor's ability to bind specific ligands, and it is possible that kepone and 4-OH-E_2 actions are mediated via the formation of such complexes.

Cross-talk between different intracellular signaling systems may also generate effects that mimic those induced by natural ligands (36–43). Thus the dogma that steroids or their mimics function only by interacting with a specific nuclear steroid receptor that serves as a transcription activating factor may not be totally reflective of *in vivo* mechanisms.

In summary, our findings in ERKO mice demonstrate that a catecholestrogen or a xenoestrogen can up-regulate the expression of an estrogen-responsive gene in the uterus via a pathway that does not appear to involve nuclear ER-α or ER-β. Because xenoestrogens and catecholestrogens are implicated in both embryo implantation (8, 44) and breast tumorigenesis (45), further characterization of this pathway will enhance our understanding of diverse steroid hormone actions in target organs.

We thank Dr. D. C. Johnson for his continuous encouragement during the course of this study. Thanks also are due to Mrs. Jue Wang for performing the *in situ* hybridization experiments, and to Kevin Day, Brian Philips, Debjani Ghosh, and Cheryl Rosenfeld for critical review of this manuscript. This work was supported in part by National Institutes of Health grants (ES 07814 to S. K. Das, HD 12304 to S. K. Dey, and ES08272 to D.B.L.), by a Breast Cancer grant from the Department of the Army (DAMD17-97-1-7171 to D.B.L.) and by an Environmental Protection Agency grant (R825295010 to D.B.L.).

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